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Plant activation is the process by which a promutagen is metabolically transformed into a mutagen by a plant system. Plants can activate promutagens into stable mutagens and these genotoxic agents may be hazardous to the environment and to the public health. This research established that a series of environmental arylamines that included *m*-phenylenediamine, 2,4-diaminotoluene, benzidine, 4-aminobiphenyl, 2-aminofluorene and 2-naphthylamine could be activated by cultured plant cells into agents that were directly mutagenic in *Salmonella typhimurium* tester strains. The mechanism of the plant-activation of these arylamines was dependent upon plant peroxidases and did not involve cytochrome P-450 mediated monooxygenation. The plant-activated products were associated with a high molecular weight fraction (>100 kDa) isolated by ultrafiltration membrane size exclusion. These products were stable (some having no reduction in their mutagenic potency after 1 yr). The plant-activated arylamine products were substrates for *Salmonella* and human *NAT2 O/N*-acetyltransferases. These products also induced unique mutant spectra at both the *hisD3052* and *hisG46* target alleles in *S. typhimurium*. Preliminary results indicate that we have isolated the plant-activated *m*PDA DNA adduct(s) from treated *S. typhimurium* cells. Finally, plant- or mammalian-activated arylamines demonstrated a synergic genotoxic response in the presence of the non-mutagenic organophosphorus ester insecticide product, paraoxon. These data suggest that plant-activated arylamines and OP-oxon products may amplify the genotoxic burden of arylamines in the environment.

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THE MECHANISMS AND EFFECTS OF THE PLANT-ACTIVATION OF CHEMICALS IN THE ENVIRONMENT: Grant Nº AFOSR-91-0432

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1 INTRODUCTION

Plants can activate promutagens into stable mutagens and these genotoxic agents may be hazardous to the environment and to the public health. Plant systems have been widely employed in classical and environmental mutagenesis. However, the environmental and human health impact of plants exposed to environmental xenobiotics were not well recognized until the presence of pesticide contaminants in food supplies caused alarm. The capability of plants to bioconcentrate environmental agents and activate promutagens into toxic metabolites is significant when one realizes the immense diversity of xenobiotics to which plants are intentionally and unintentionally exposed. Finally, we all must be attentive to the effects that toxic agents may have on the biosphere and the grave global consequences that would result in a disruption in the carbon cycle. Plant activation is the process by which a promutagen is metabolically transformed into a mutagen by a plant system. In mammalian systems the majority of enzymes participating in oxidative desulfuration, dealkylation, epoxidation, or ring hydroxylation involve cytochrome P-450-type monooxygenases. It is unknown if microsomal cytochrome P-450 in plants have enzymatic characteristics similar to those of mammalian liver. The optical and magnetic properties of plant cytochrome P-450 are similar to those of hepatic microsomes. Although limited data exist about the inducibility of plant cytochrome P-450, it is unknown if there is an equivalent inducible system to hepatic monooxygenases. Plant peroxidases catalyze the oxidation of a diverse class of xenobiotics. Peroxidases are ubiquitous in plants, however, only limited data are available that demonstrate their participation in the *in vivo* metabolism of foreign compounds.

1.1 Background and Significance

1.1.1 Involvement of Plant Activation in Genetic Toxicology

Attention to toxic plant metabolites of environmental xenobiotics has grown since our first demonstration of *in vitro* plant activation (for a review see Plewa and Wagner, 1993). However, the environmental and human health impact of plants exposed to xenobiotics were not well recognized until the presence of pesticide contaminants in food supplies caused alarm. The capability of plants to bioconcentrate environmental agents and activate promutagens into toxic metabolites is significant when one realizes the immense diversity and quantities of foreign compounds to which plants are exposed. Finally we must all be concerned as to the effects toxic agents may have on the biosphere and the grave global consequences that would result in a disruption in the carbon cycle. A partial list of chemicals that have been identified as plant-activated promutagens is presented in Table 1.

Table 1. List of Chemical Promutagens Activated by Plant Systems (For references see Plewa and Wagner, *Annual Review of Genetics*, 27:93-113, 1993)

Studies on Intact Plants or Plant Cells with Genetic Endpoints		
N-Acetylaminofluorene	Pesticides:	2-Aminofluorene
Aflatoxins	Chlordane	Trp-P-2
Aniline	1,2-Dibromo-	Glu-P-1
m- o- p-Aminophenol	ethane	Benzidine
Benzo[α]pyrene	Heptachlor	Ethanol
Furylfuramide	Maleic hydrazide	*N*-Nitrosamines
Pyrrolizidine alkaloids	*s*-Triazines	Phenol
		Sodium azide
Studies Using Plants, Tissues or Cells with External Genetic or Biochemical Endpoints		
2-Aminofluorene	Pesticides:	4-Aminobiphenyl
Benzidine	Alachlor	Benzo[α]pyrene
Cyclophos-	Chlordane	2,4-Diaminotoluene
phamide	Heptachlor	2-Naphthylamine
Ethanol	Propachlor	Sodium azide
4-Nitro-*o*-phenylenedi-	*s*-Triazines	*m- o*-Phenylenediamine
amine		
Studies on Plant Cell-Free Systems		
N-Acetylaminofluorene	Pesticides:	Aflatoxin B_1
Aniline	Atrazine	2-Aminofluorene
Benzo[α]pyrene	Captan	Cyclophosphamide
7,12-Dimethyl-benz-	Chlordane	Ethanol
[α]anthracene	1,2-Dibromo-	Pyrenes
4-Nitro-*o*-phenylenedi-	ethane	
amine	Diquat	
m- o-Phenylenediamine	Maleic hydrazide	
Sodium azide	Niclofen	
	Pentac	
	Ziram	

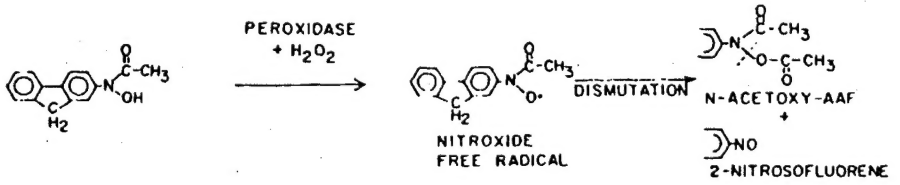
1.1.2 Oxidative Metabolism in Higher Plant Systems

In mammalian systems nearly all enzymes participating in oxidative desulfuration, dealkylation, epoxidation, or ring hydroxylation are microsomal in nature (equation 1). Currently it is unknown if microsomal cytochrome P-450 in plants have enzymatic characteristics similar to those of mammalian liver. Although the optical and magnetic properties of plant cytochrome P-450 are similar to those of hepatic microsomes, plant cells generally contain very little cytochrome P-450 (Higashi, 1988). Although limited data exist about the inducibility of plant cytochrome P-450, it is unknown if there is an equivalent inducible system to hepatic monooxygenases.

$$R\text{-}NH_2 + O_2 + NADPH{+}H^+ \rightarrow R\text{-}NHOH + H_2O + NADP^+$$

(1) The overall reaction in the monooxygenation of an aromatic amine by the cytochrome P-450 enzyme system.

Plant peroxidases catalyze the oxidation of a diverse class of xenobiotics (equation 2). Higashi (1988) described two types of oxidative reactions in which plant peroxidases are participants. The first type is the peroxidative reaction that requires H_2O_2; the second type uses O_2. Although peroxidases are ubiquitous in plants, their participation in the *in vivo* metabolism of foreign compounds is speculative at this time (Kuwahara et al., 1988). Recently we discovered that phenylenediamine-induced mutation in *Tradescantia* was suppressed by peroxidase inhibition under *in vivo* conditions (Gichner et al., 1994).



(2) Peroxidase-catalyzed oxidation of *N*-hydroxy-2-acetylaminofluorene to the nitroxide free radical after dismutation results in 2-nitrosofluorene and *N*-2-acetoxy-acetylaminofluorene.

1.1.3 Studies in Plant Activation Using Aromatic Amines as Model Promutagens

1.1.3.1 Aromatic Amines

Aromatic amines are classical promutagens and procarcinogens and have been used to resolve questions involving mutagenic activation, cancer induction and human polymorphic sensitivity to environmental agents (Weisburger, 1988). These agents are substrates for mammalian activation by the cytochrome P-450-mediated pathway as well as by cellular peroxidases such as prostaglandin H synthase (Donaldson and Luster, 1991; Guengerich et al., 1988). 2-Aminofluorene, N-acetylaminofluorene benzidine, isomers of phenylenediamine, Glu-P-1 and Trp-P-2 induce chromosome aberrations and point mutations in intact plants or cultured plant cells. We use aromatic amines as a class of model compounds to study the mechanisms of plant activation as well as the molecular effects of the plant-activated products (Plewa and Wagner, 1993).

1.1.3.2 The Plant Cell/Microbe Coincubation Assay

This assay was developed in our laboratory and employs living plant cells in suspension culture as the activating system and specific microbial strains as the genetic indicator organism (Plewa et al., 1983; Plewa et al, 1988; Wagner et al., 1994). The plant and microbial cells are coincubated together in a suitable medium with a promutagen. The activation of the promutagen is detected by plating the microbe on selective media; the viability of the plant and microbial cells may be monitored as well as other components of the assay (Figure 1).

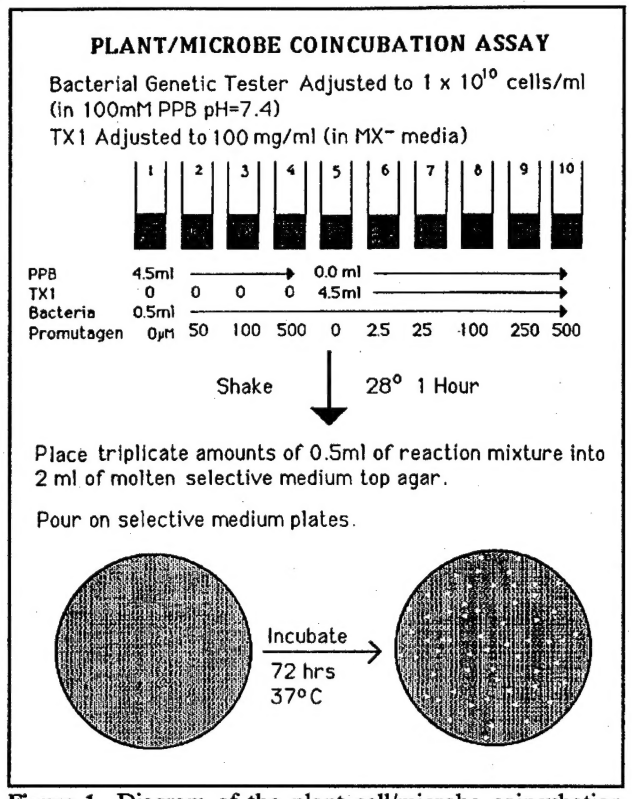


Figure 1 Diagram of the plant cell/microbe coincubation assay.

In general the assay involves the following description. Long-term plant cell suspension cultures of tobacco (*Nicotiana tabacum*) TX1, are maintained in MX medium. Individual *S. typhimurium* strains — or other microbial cells with specific genetic endpoints — are used as the genetic indicator organisms. A TX1 cell culture is grown at 28°C to early stationary phase; the cells are washed and suspended in MX^- medium. MX^- medium lacks plant growth hormone. The fresh weight of the plant cells are adjusted to 100 mg/ml, and the culture is stored on ice (≤30 min) until used. An overnight culture of *S. typhimurium* is grown from a single colony isolate in 100 ml of Luria broth at 37°C with shaking. The bacterial suspension is centrifuged and washed in 100 mM potassium phosphate buffer, pH 7.4. The titer of the suspension is determined spectrophotometrically, adjusted to 1×10^{10} cells/ml, and the culture stored on ice. In the coincubation assay, each reaction mixture consists of 4.5 ml of the plant cell suspension in MX^- medium, 0.5 ml of the bacterial suspension (5×10^9 cells), and a known amount of the promutagen in μl amounts. Concurrent negative controls consist of plant and bacterial cells alone, heat-killed plant cells plus bacteria and the promutagen, and both buffer and solvent controls. These components are incubated at 28°C for 1 h with shaking at 150 rpm. After the treatment time, the reaction tubes are placed on ice. Triplicate 0.5 ml aliquots (~5×10^8 bacteria) are removed and added to molten top agar supplemented with 550 μM histidine and biotin. The top agar is poured onto Vogel Bonner (VB) minimal medium plates, incubated for 72 h at 37°C, and revertant *his*$^+$ colonies are scored (Maron and Ames, 1983). The remainder of the reaction mixture may be used to determine the viability of the plant and bacterial cells. One

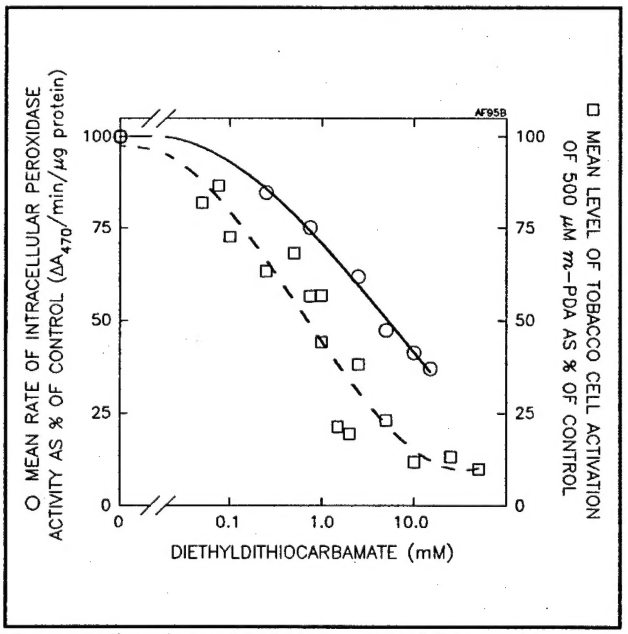


Figure 2 The relationship between the inhibition of TX1 cell peroxidase activity (O) and the repression of TX1 activation of *m*-phenylenediamine (□) versus the log concentration of diethyldithiocarbamate.

volume of cold 250 mM sodium citrate buffer, pH 7, is added to each reaction tube and 0.5 ml of this suspension is removed and mixed with 2 ml of MX^{-} medium. The viability of the TX1 cells is immediately determined using the phenosafranin dye exclusion method. The viability of the bacterial cells may be determined by adding 1 ml of the cold reaction mixture to 1 ml of cold 100 mM phosphate buffer, pH 7.4. After a dilution series in phosphate buffer is conducted a specific volume is added to each of three molten LB top agar tubes and poured upon LB plates. After incubation at 37° C for 24 h, the viable bacterial cells grow into discrete colonies and are counted.

1.1.3.3 Inhibitors to Identify Biochemical Pathways in Plant Activation

The use of enzyme inhibitors for specific pathways has aided the biochemical characterization of the metabolism of xenobiotics in mammalian and plant species. With inhibition studies care must be taken to insure that the biological competency of the assay is not compromised. Seven inhibitors — diethyldithiocarbamate, metyrapone, 7,8-benzoflavone, potassium cyanide, (+)-catechin, methimazole and acetaminophen — were incorporated in the plant cell/microbe coincubation assay. These chemicals have been used to study metabolism in animal systems as well as in plants. Using these inhibitors, plant cell peroxidases were found to be involved in the activation of the promutagenic aromatic amines, 2-aminofluorene and *m*-phenylenediamine by tobacco cells (Wagner et al., 1989; 1990). One of the inhibitors, diethyldithiocarbamate repressed the activation of these promutagens by inhibiting the activity of tobacco cell peroxidase (Figure 2) (Plewa et al., 1991).

1.1.3.4 Isolation of High Molecular Weight Plant-Activated Aromatic Amine Products

Vacuoles and the cell wall are sites of deposition of many endogenous plant conjugates. Deposition is a detoxification mechanism. However, under suspension cell culture conditions, plant cells preferentially transported xenobiotic metabolites into the medium (Gareis et al., 1992). The tobacco cell-activated mutagenic products of 2-aminofluorene, *m*-phenylenediamine, benzidine, and 4-aminobiphenyl were isolated from the cell culture medium (Ju and Plewa, 1993; Plewa et al., 1993; Seo et al., 1993). The molecular weight limits were determined by membrane ultrafiltration. The products of these aromatic amines were isolated in a fraction of 100–1,000 kDa (Figure 3). These high molecular weight products are not found in the mammalian metabolism of aromatic amines. These products were not toxic to the plant cells and were very stable, retaining their mutagenic potency for over 1 year (Seo et al., 1993). There seems to be an incongruity in that plant cells generate stable, mutagenic products from aromatic amines that are not toxic to the plant cells themselves. This may be resolved by the fact that the plant-activated products are proximal mutagens and must be further metabolized into the ultimate mutagenic agents.

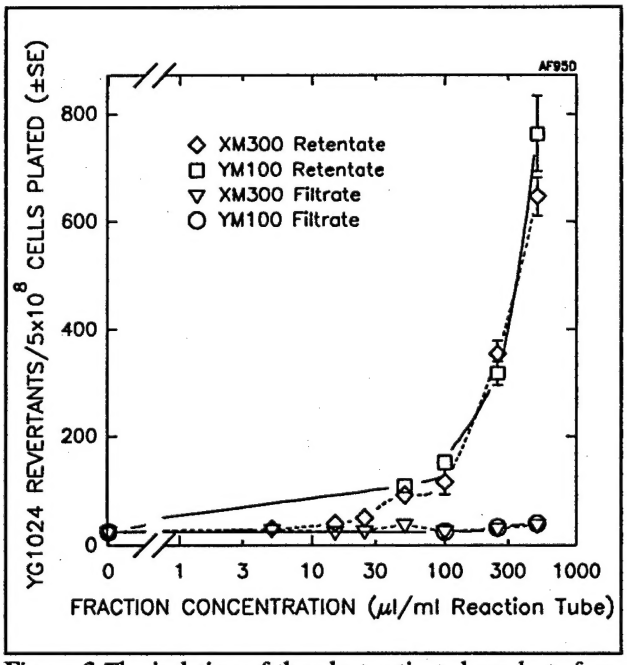


Figure 3 The isolation of the plant-activated products from TX1 cells treated with 500 μM *m*-phenylenediamine in the high molecular weight fraction after ultrafiltration membrane chromatography. The fractions that demonstrated the highest mutagenic activity were those that contained high MW molecules (◇ ≥300 kDa; □ ≥100 kDa). Little mutagenic activity was seen in the lower MW fractions isolated from the treated TX1 cells (▽ < 300 kDa; ○ < 100 kDa).

1.1.3.5 Plant-Activated Aromatic Amine Products as Substrates for Acetyltransferases

The first step in the mammalian hepatic activation of aromatic amines is *N*-hydroxylation. Cellular acetyltransferases are essential in further metabolizing *N*-hydroxyarylamines to their ultimate mutagenic products (Saito et al., 1985). To study the effect of acetyltransferase activity on the plant-activated aromatic amine products we used a series of *S. typhimurium* strains that differentially express acetyl-CoA: *N*-hydroxyarylamine *O*-acetyltransferase (OAT) (Wagner et al., 1994). The plant-activated products of 2-aminofluorene, benzidine, *m*-phenylenediamine, 4-aminobiphenyl, 2,4-diaminotoluene and 2-naphthylamine served as substrates for bacterial OAT and induced mutation in *Salmonella.* A direct relationship existed between the mutagenic potency of the plant-activated product and the amount of OAT expressed in the genetic indicator cells. The data for benzidine is presented in Figure 4. The strain that has the highest expression of OAT (YG1024) is the most sensitive to the plant-activated benzidine product.

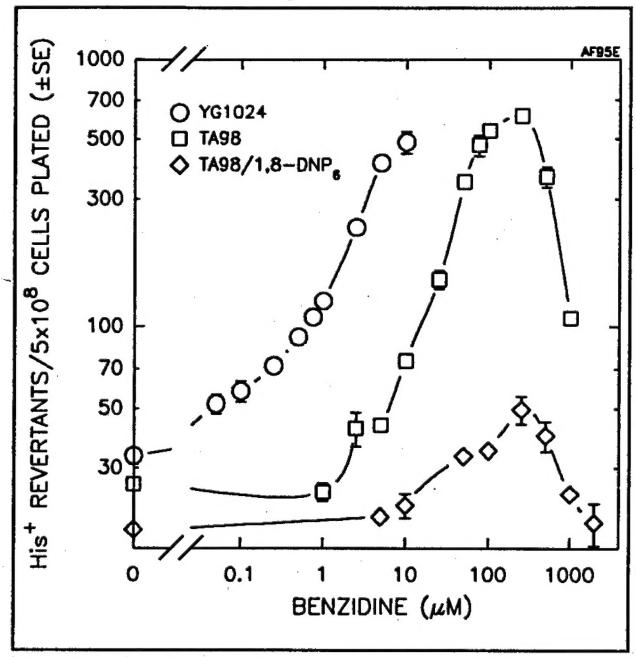


Figure 4 The effect of the expression of OAT in the genetic indicator cells exposed to the plant-activated products of benzidine. *S. typhimurium* strain TA98/1,8-DNP$_6$ does not express *OAT*, while TA98 and YG1024 express the wild-type level and over-express *OAT*, respectively.

The N-terminal region of *S. typhimurium O*-acetyltransferase and the corresponding region of *N*-acetyltransferases from human, rabbit, hamster and chicken expressed 25-33% homology. Among the enzymes compared, a highly conserved region with the amino acid sequence of Arg-Gly-Gly-X-Cys, is essential for the acetyl-coenzyme A-binding site (Dupret and Grant, 1992; Watanabe et al., 1992). Thus these enzymes are closely related in their structure and function.

NAT1 and *NAT2* are two human genes that encode functionally distinct but similar cytosolic CoASAc:arylamine *N*-acetyltransferase (Dupret and Grant, 1992). Although *NAT1* and *NAT2* have diverse substrate specificities, they share over 80% amino acid sequence homology (Blum et al., 1990). Rat hepatic S9 activation of 2-aminofluorene, benzidine and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline was examined in *S. typhimurium* strains containing human *NAT1* and *NAT2*, DJ400 (TA1538/1,8-DNP:pNAT1) and DJ460 (TA-1538/1,8-DNP:pNAT2), respectively. All three aromatic amines were mutagenic in DJ460. Only 2-aminofluorene and benzidine were mutagenic in DJ400 (Grant et al., 1992). Plant-activated 2-aminofluorene, benzidine and 4-aminobiphenyl products also are substrates for human NAT1 and NAT2 (Figure 5) (Ju and Plewa, 1993; Seo and Plewa, 1993).

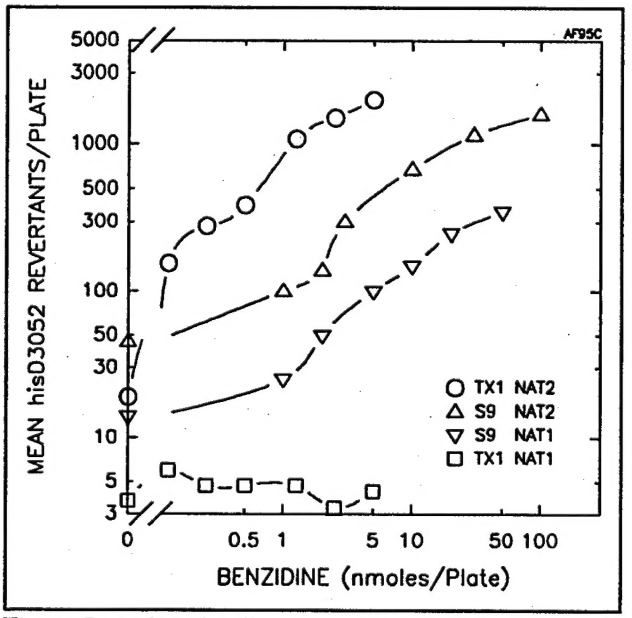


Figure 5 Log/log plot illustrating the mutagenic response of rat hepatic microsomal S9-activated benzidine assayed in *S. typhimurium* that expressed human NAT1 (▽) or NAT2 (△) (from Grant et al., 1992). Mutagenic response of plant-activated benzidine in strains that expressed human NAT1 (□) or NAT2 (○) (preliminary studies Plewa et al., 1994).

1.1.3.6 Current Working Model for the Plant-activation of Aromatic Amines

We prepared a model of the TX1 cell activation of *m*-PDA that we extend to the plant-activation of aromatic amine promutagens (Seo et al., 1993). The model (Figure 6) — albeit simplistic and incomplete — integrates our data into a mechanistic framework and serves as a foundation for new experimental designs. The model has seven components. They are (1) the aromatic amine ($R-NH_2$) is transported into the plant (TX1) cell, (2) TX1 intracellular peroxidase oxidizes the molecule (R–NHOH), (3) the metabolite is conjugated to a macromolecule (RN-HOH-conjugate), (4) the amine-conjugate is secreted into the extracellular medium (the spent medium also has a limited capacity to activate the aromatic amine promutagen), (5) the conjugate or a deconjugated plant-activated metabolite is absorbed by the tester strains, (6) the plant-activated *N*-hydroxylated product is acetylated ($R-NHO-COCH_3$) and deacetylated by the bacterial acetyl-CoA: *N*-hydroxyarylamine *O*-acetyltransferase, and (7) the deacetylation results in a highly reactive nitrenium ion ($R-NH^+$).

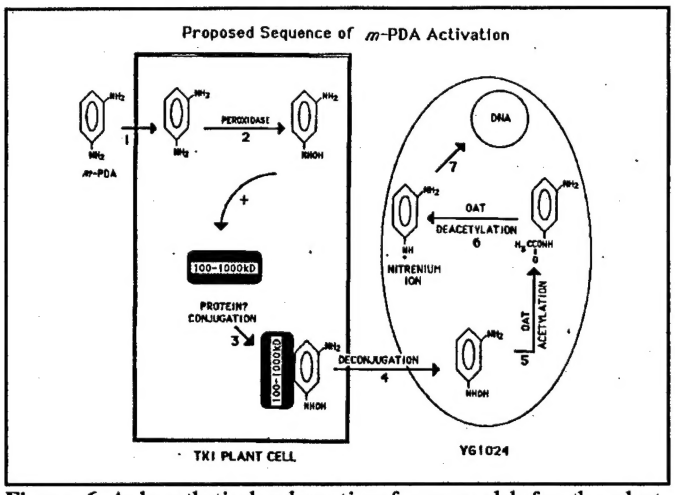


Figure 6 A hypothetical schematic of our model for the plant-activation of *m*-phenylenediamine.

1.1.3.7 Molecular Effects of Plant-Activated Promutagens

The molecular effects of the plant-activated products of promutagens is virtually unknown. One study reported the induction of nuclear DNA adducts by benzo[α]pyrene in a suspension culture of *Echinacea purpurea*; however these adducts were not characterized (Rether et al., 1990).

Mutant spectra analysis has been conducted on many of the *S. typhimurium* tester strains (Bell et al., 1991; Cebula and Koch, 1990; Kupchella and Cebula, 1991). These studies define the molecular basis of both spontaneous and induced revertants. In the *hisD3052* allele a high frequency of reversion occurs by a CG/GC deletion which is located in an alternating CG octamer (D878-885); a colony-probe hybridization procedure was developed to detect this -2 deletion (Cebula and Koch,1990; Kupchella and Cebula, 1991). Revertants at the *hisD3052* allele of strains YG1024, TA98, and TA98/1,8-DNP_6 were collected after coincubation with tobacco cells and 2-aminofluorene (Plewa et al., 1995). 40% of the spontaneous TA98 revertants were due to -2 events at D878-885. In contrast, 94% of the plant-activated 2-aminofluorene-induced TA98 revertants probed as this -2 deletion. Cebula and Koch (1990) found that 92% of the revertants induced by mammalian S9-activated acetylaminofluorene were a -2 deletion in this hotspot region. YG1024 was more sensitive than TA98 to the plant-activated metabolites of aromatic amines due to elevated levels of *O*-acetyltransferase. 98% of the induced YG1024 revertants were the result of a -2 event. Despite the difference in mutagenic sensitivity, there was no difference in the frequency of -2 deletions at D878-885 of these plant-activated 2-aminofluorene-induced revertants.

1.1.4 Background Summary

This review is limited to a specific area in the field of environmental mutagenesis; its significance is based on the fact that plants serve as the foundation of the food chain. It is disconcerting that plants can activate promutagens and store the products in forms that may induce mutations in consuming organisms. Only recently have we become cognizant of the impact of plants in their role of recycling xenobiotics in the biosphere. The capacity of plants to serve as a global "green sink" for xenobiotics is

unknown. Since the biosphere is a closed system, research on the mechanisms, biological actions and fate of plant metabolized xenobiotics is a priority.

2 RESULTS AND DISCUSSION FOR SPECIFIC PROJECTS SINCE THE LAST INTERIM REPORT

2.1 Mutant Spectra Analysis at *Hisg46* in *Salmonella typhimurium* Induced by Mammalian S9- and Plant-Activated Benzidine

2.1.1 Introduction

Mutation spectra analysis was conducted on *S. typhimurium* tester strains to define the molecular basis of both spontaneous and induced revertants. A set of *S. typhimurium* strains was developed by Watanabe et al., (1990) which have a high expression of acetyl-CoA:*N*-hydroxyarylamine *O*-acetyltransferase (OAT). Strain YG1029 (TA100, pYG219) has an enhanced sensitivity to plant- and mammalian-activated products of benzidine as compared to TA100.

We demonstrated that benzidine was activated by plant cells (Ju and Plewa, 1993; Figure 7). Benzidine, an aromatic amine, is a promutagen and procarcinogen and a substrate for mammalian activation by the cytochrome P-450-mediated pathway (Figure 8). Mutation spectra analysis was conducted on the *hisG46* allele of *S. typhimurium* strain YG1029 from spontaneous revertants and revertants induced by the plant and mammalian (S9) activation of benzidine. The metabolite(s) of plant-activated benzidine were isolated in an XM300 retentate fraction using Amicon ultrafiltration.

The objectives of this research were to construct and compare the mutation spectra of the YG1029 revertants induced by both plant- and mammalian-activated benzidine metabolite(s). These data were compared with spectra generated from spontaneous YG1029 and TA100 revertants.

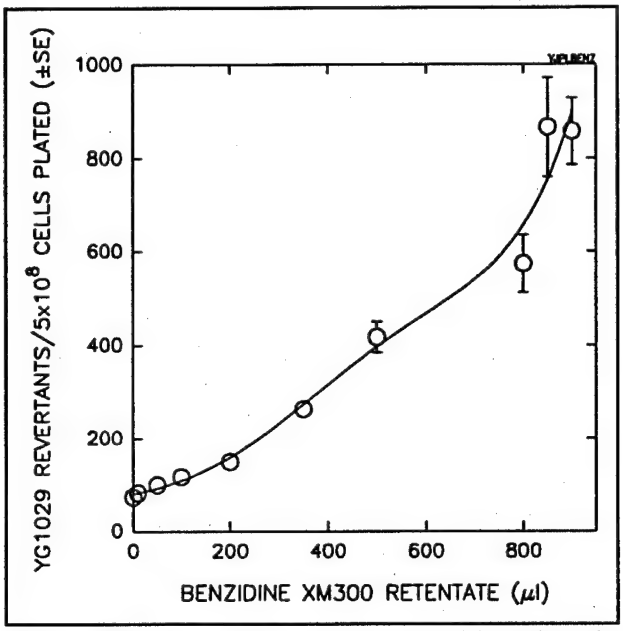


Figure 7 The mutagenicity of plant-activated benzidine: the XM300 fraction.

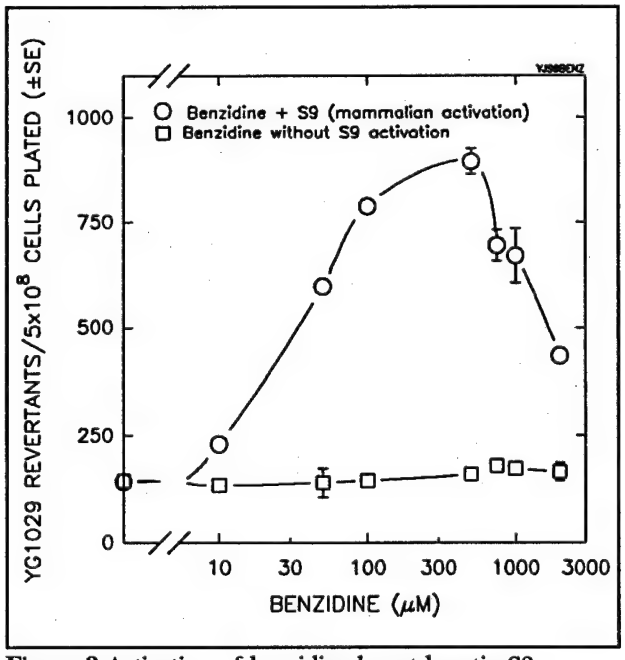


Figure 8 Activation of benzidine by rat hepatic S9.

2.1.2 Methods

2.1.2.1 Revertant Collection

For the collection of independent spontaneous *his*$^+$ revertants, single colony isolates were collected from master plates and each grown in 1 ml of LB (+ antibiotics) liquid medium overnight at 37°C. 100 µl of each suspension was added to VB minimal media molten supplemented top agar, poured onto a VB plate and incubated for 72 h. One *his*$^+$ colony was isolated per plate and inoculated into VB+biotin liquid medium and grown overnight. From each

culture, cells were streaked onto a VB+biotin quartet plate, and grown overnight. The quartet plates were stored at 4° C.

For the collection of the plant-activated benzidine-induced revertants, TX1 cells (*Nicotiana tabacum*), tobacco suspension culture, were treated with 50 μM benzidine for 3 h while shaking at 28° C. The cells were removed from the medium by centrifugation. The supernatant was recovered and centrifuged at 100,000 × g for 3 h at 4° C. Under these conditions molecules >1,000 kDa should sediment. The ultracentrifuged supernatant was fractionated using a XM300 ultrafiltration membrane which has a cut-off point of 300 kDa. The retentate was used for treating the bacteria. Reaction tubes contained 1×10^9 YG1029 cells, 100 mM phosphate buffer, pH 7.4 and from 0.5-90% of the XM300 retentate for a total volume of 1 ml. The reaction tubes were incubated for 1 h at 37° C and plated on minimal medium plates. Plates were chosen that yielded an approximately 10-fold increase in mutation over the spontaneous frequency. 50 single colonies were collected from each plate, streaked onto VB+biotin quartet plates, and stored at 4° C.

For the collection of the S9-activated benzidine-induced revertants, reaction tubes contained benzidine from 10 μM to 2 mM, 100 mM phosphate buffer, pH 7.4, 1×10^9 YG1029 cells, and 50% (v/v) of S9 mix. The reaction tubes were incubated for 1 h at 37° C and plated on minimal medium plates. Concentrations of benzidine were chosen that yielded approximately an 8-9× increase in mutation over the spontaneous frequency with no toxicity to the bacteria cells . 50 single colonies were collected from each plate chosen as described previously. 300 spontaneous revertants and 300 each of induced revertants by plant- and mammalian-activated benzidine were analyzed. Revertant colonies from the quartet plates were grown in VB+biotin liquid media overnight at 37° C prior to DNA extraction.

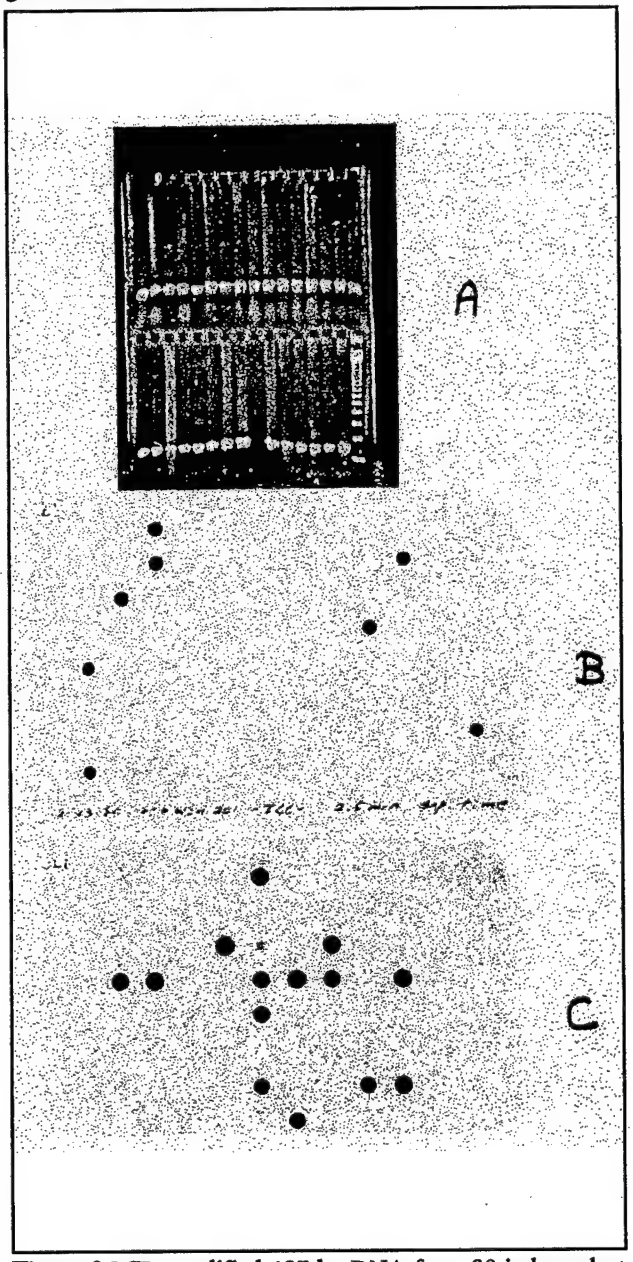


Figure 9 PCR-amplified 187 bp DNA from 30 independent *hisG46* revertants induced by plant-activated benzidine product visualized on a 2% agarose gel. The lower middle lane is the no DNA control and the lane in the lower right corner is the DNA size standard (panel A). Dot blot analysis of the membranes containing the PCR-amplified DNA using two specific probes for specific base pair substitution mutations [panel B (TCC) and panel C (ACC)].

2.1.2.2 Genomic DNA Isolation, PCR Amplification and Hybridization

Genomic DNA was extracted using a minipre paration procedure, the DNA was suspended in 100 μl TE buffer, pH 7.4 and stored at -20° C. A 187 base pair DNA fragment containing the *hisG46* region was amplified for each genomic DNA sample using PCR. A 10-fold dilution of the genomic DNA was used as a template. The primers used were HISG46A (5′-GCCTGATTGCGATGGCGG-3′) and HISG46B (5′-GTCAAGACGGCGCTGGG-3′). The concentration of primers was 0.2 μM. The thermal cycler was programmed for an initial step at 94° C

for 5 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 30 sec. The PCR product was analyzed by 2% agarose gel electrophoresis to verify the amplification of the correct region (Figure 9A). The PCR product was transferred to a charged nylon membrane using a Bio-Dot micro-filtration apparatus. Each well of a 96-well microtiter plate was filled with 195 µl of buffer (TE pH 7.4, 400 mM NaOH, 10 mM EDTA) and 5 µl of PCR product. The plate was heated to 95°C for 10 min. The contents from each well were transferred to corresponding wells on the dot blot apparatus. Using vacuum, each sample was drawn onto a charged nylon membrane. 500 µl of 400 mM NaOH was added to each well and pulled through the membrane by vacuum. The membrane was removed from the apparatus, rinsed in 2× SSC, and exposed to UV radiation to cross-link the DNA. The membrane was allowed to air dry and was stored at room temperature. The *hisG46* allele is a missense mutation (CTC → CCC) at *hisG* codon 69 which renders the cell *his*$^-$. In five of the six possible base-pair substitutions at the first or second residue of the codon the cell reverts to histidine prototropy (Table 2). Also the occurrence of revertants expressing a CCC sequence at codon 69 indicates they are suppressor mutants. Thus, six possible events account for all of the *hisG46* revertants. A modified version of the Amersham nonradioactive ECL3′-oligolabelling and detection system was used to identify each of the six possible base-pair substitution mutations. Each probe was end-labelled with fluorescein-dUTP. Each membrane was pre-hybridized for 30 min at 34°C with no probe. Fluorescein-dUTP-labelled probe was added and incubated for 1 h at 34°C. The membranes were washed twice in 5 × SSC, 0.1% SDS for 5 min at 34°C followed by two washes in 0.9 × SSC, 0.1% SDS for 15 min at 39°C. The membranes were incubated for 30 min in block solution, rinsed and incubated for 30 min with anti-fluorescein horseradish peroxidase conjugate. Following extensive washing, the membranes were exposed for 5 min on X-ray film.

Table 2. Oligonucleotide probes used to detect reversion events at *hisG46* in *S. typhimurium.*

Reversion Event	Probe
CCC→ACC	5′—GTCGATACCGGTTAT
CCC→TCC	5′—GTCGATTCCGGTTAT
CCC→GCC	5′—GTCGATGCCGGTTAT
CCC→CTC	5′—GTCGATCTCGGTTAT
CCC→CAC	5′—GTCGATCACGGTTAT
CCC→CCC[1]	5′—GTCGATCCCGGTTAT

[1]Reversion due to an extragenic suppression mutation.

All mutants containing the base-pair substitution identical to the probe appear as dark dots (Figure 9, B and C). The dots which are absent indicate any of the other five events. Each membrane had six controls in the upper left corner representing each of the six possible base-pair substitutions. Only the control matching the probe should appear as a dark dot. After each hybridization procedure, the membranes were stripped of all labelled probe by first washing in 5 × SSC, 0.1% SDS for 5 min at room temperature followed by 0.1 × SSC, 0.1% SDS for 1 h at 65°C and a final wash in 5 × SSC, 0.1% SDS for 5 min at room temperature. The membranes were wrapped in plastic wrap and stored at -20°C. The membrane was rehybridized with all six probes without any loss in specificity.

2.1.3 Results and Discussion

Of the YG1029 spontaneous revertants, transition mutations were 31.8% and transversion mutations were 68.2% (Table 3). The spontaneous mutation spectrum differs significantly from TA100 with 50.5% of revertants from transition and 49.5% from transversion mutations (Cebula and Koch, 1990).

Table 3. Percent frequencies of each type of reversion event at *hisG46*.

Revertants at *hisG46*	Transitions		Transversions			Extragenic Suppressor
	TCC	CTC	ACC	CAC	GCC	CCC
YG1029 Spontaneous	9.8	22.0	17.3	48.2	2.7	0
TX1 Activated Benzidine-Induced YG1029	7.1	15.4	16.0	59.2	2.4	0
S9 Activated Benzidine-Induced YG1029	3.9	10.5	15.4	68.0	2.4	0
TA100 Spontaneous	11.0	39.0	16.0	30.0	3.0	1

According to the mutation spectra results (Table 3) there are more transversion mutations in YG1029 than in TA100. With plant-activated benzidine products, 22.5% of the recovered revertants resulted from transitions and 77.6% from transversions. The revertants induced by S9- activated benzidine were 14.4% transition and 85.8% transversion mutations. Comparing the mutant spectra of spontaneous, plant-activated and S9-activated benzidine, there is a significant difference among these three spectra. When comparing the distribution of the transition mutations the plant-activated spectra differed not only from the control but also from the S9-activated benzidine-induced YG1029 mutants (Figure 10). The different yields of the mutations (CCC → CTC and CCC → CAC) resulted in the difference between spontaneous and induced revertants. There also was a significant difference between the YG1029 and TA100 spontaneous mutant spectra.

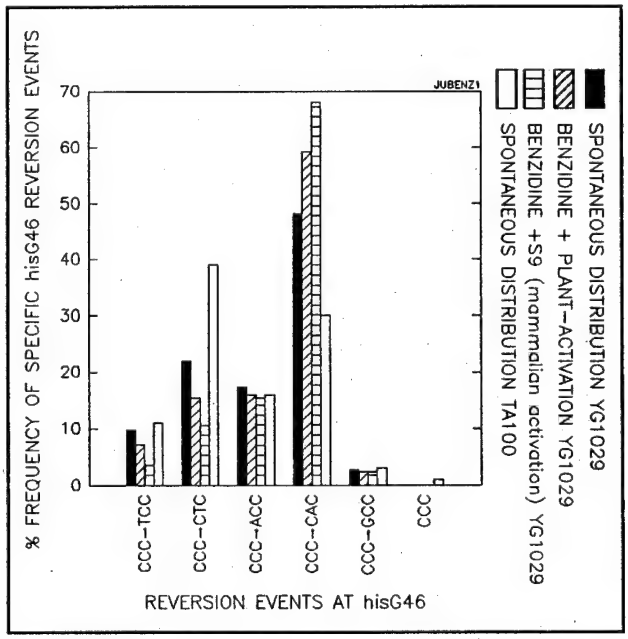


Figure 10 Distribution of reversion events at *hisG46* in *S. typhimurium* TA100 and YG1029.

Table 4. Chi-Square analysis of mutation spectra at the *hisG46* allele						
Comparisons	χ^2 (Tv +Ts)	*P*	χ^2 Tv	*P*	χ^2 Ts	*P*
YG1029						
TX1-BZ vs. Spontaneous	5.36		2.64		2.72*	0.025<*P*<0.05
S9-BZ vs. Spontaneous	17.93*	*P*<0.001	8.37*	0.005<*P*<0.01	9.61*	*P*<0.001
S9-BZ vs. TX1-BZ	4.33		1.33		3.00*	0.025<*P*<0.05
TA100 spontaneous vs. YG1029 spontaneous	20.39*	*P*<0.001	7.00*	0.025<*P*<0.05	13.29*	*P*<0.001

*: There is a significant difference between the two groups.
Tv: Transversion mutation
Ts: Transition mutation
TX1-BZ: TX1-activated benzidine-induced revertants
S9-BZ: S9-activated benzidine-induced revertants

This study illustrates that the introduction of the plasmid pYG219 into *S. typhimurium* enhanced CCC → CAC transversion mutations and reduced CCC → CTC transition mutations. According to the Chi-square analysis (Table 4), the mammalian-activated benzidine-induced mutation spectrum differed from the spontaneous mutant spectrum. Although a significant difference among these spectra existed at the level of transition mutations, the global distribution of the plant-activated benzidine-induced mutation spectrum was not different from the spontaneous or the S9-activated benzidine-induced mutation spectra.

2.2 ^{32}P Postlabelling of DNA Adducts Isolated from *S. typhimurium*

S. typhimurium cells was treated with plant-activated *m*PDA at a concentration that induced the highest mutant yield (YG1024 revertants/5×10^8 cells treated). The treatment continued for 3 h followed by a wash in 1 volume phosphate buffer. An aliquot of the suspension (5×10^8 cells) was added to supplemented VB top agar and plated onto VB medium to verify the mutagenic response. The rest of the cells were harvested, lysed by lyticase, and genomic DNA was isolated and extracted sequentially with phenol/chloroform/isoamyl alcohol (25:24:1), and 1 volume chloroform/isoamyl alcohol (24:1). The DNA was ethanol precipitated and sequentially incubated with RNase T1 and proteinase K. The DNA was further extracted, ethanol precipitated and stored in succinate buffer. The DNA was quantitated by absorbance at 260 nm. For each sample, 2-5 μg genomic DNA was digested for 3 h at 37° C in succinate buffer (pH 6) with 10 mM $CaCl_2$, 0.05 U spleen phosphodiesterase II and 2.25 U micrococcal nuclease in 20 μl total volumes. The arylamine adducts was enriched using butanol extraction and the extract was dried in a Speed-Vac and stored at −80° C.

15μl of water was added to each DNA adduct extract plus 5 μl of labelling mix. For each 5 μl aliquot the labelling mix consisted of 2.5 μl 10× buffer (100 mM Bicene, 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM spermidine, pH 9), 7 U polynucleotide kinase and 20-25 μCi [γ-^{32}P]ATP (6,000 Ci/mmol). The kinase reaction was conducted for 35 min at 37° C after which 100 mU apyrase was added to the mixture and incubated an additional 30 min.

A Beckman high performance liquid chromatograph with an analytical RP-C18 column (5 μm Ultrasphere C18 column (4.6 mm × 150 mm) plus a guard column) was used to separate the ^{32}P-labeled nucleotide-arylamine adducts. The mobile phase and initial gradient condition was 30 mM KPO_4 (pH 6.0) containing 5 mM tetrabutylammonium phosphate and 90% CH_3CN for 10 min followed by 10% CH_3CN increasing to 50% for 50 min at the flow rate of 1.5 ml/min. The HPLC eluate was collected via a fraction collector and the radioactivity of each 1.5-ml fraction was assayed directly, without scintillation cocktail, for ^{32}P-labeled agents by Cerenkov counting in a Beckman scintillation spectrometer (Beckman, LS 6000IC). An example illustrating *m*PDA-DNA adducts isolated from genomic *S. typhimurium* YG1024 DNA is presented in Figure 11. These data clearly show at least four (and possibly more) *m*PDA-DNA adducts isolated from treated cells. All samples were stored at -20° C for future structural analysis.

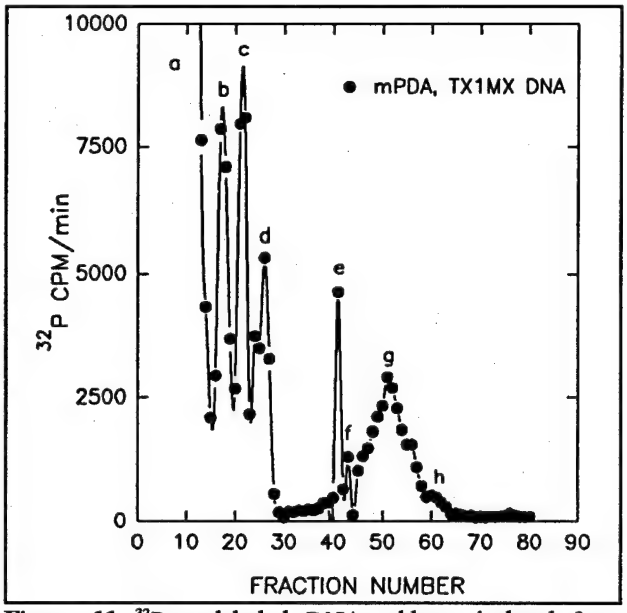


Figure 11 ^{32}P-postlabeled DNA adducts isolated from YG1024 cells treated with plant-activated *m*PDA. Peaks a, b, c, d, are from ^{32}P-ATP, and postlabeled nucleotide diphosphates, peaks e, f, g, h are from postlabeled presumptive *m*PDA-DNA adducts (Plewa 1995, unpublished data).

2.3 A Sensitive HPLC Method to Determine Acetyl-COA:*N*-hydroxyarylamine *O*-Acetyltransferase in *Salmonella* Tester Strains

2.3.1 Introduction

Many aromatic amines are promutagens which can be activated into potent frameshift mutagens by cultured tobacco cells (TX1) with *S. typhimurium* tester strains as the genetic indicator organisms. Acetyl CoA:*N*-hydroxarylamine *O*-acetyltransferase (OAT) has been shown to play a key role in the activation of promutagenic aromatic amines. Recently, new *Salmonella* tester strains harboring human *N*-acetyltransferase (NAT) have been developed. Even though both types of acetyltransferase catalyze the same type of enzymatic reactions, they exhibit different substrate specificities. To investigate the relationship between the mutagenic potency of the plant-activated metabolites of aromatic amines and the level of OAT or NAT activity among these bacterial tester strains, the acetyltransferase activity of each strain was determined from cell-free extracts. Acetyltransferase activity was determined by incubating the cell-free extract from each strain with 2-aminofluorene (2-AF) as the acetyl group acceptor and acetyl CoA as the acetyl group donor. The resulting product, acetyl-2-AF(2-AAF) was monitored with an HPLC.

2.3.2 Methods

2.3.2.1 Cell Free Extract Preparation

Each of the *S. typhimurium* strains were grown in LB medium to early stationary phase. They were then washed in 100 mM PPB (pH 7.4) and suspended in an extraction buffer (50 mM Tris-HCl pH 7.5, 1 mM DTT). The cells were disrupted by pulse-sonication and the cell-free extract fraction was collected after centrifuging the cell suspension at 10,000 g for 10 min at 4° C. The protein concentration of the cell-free extract (CFX) from each strain was determined with the Bio-Rad protein assay.

2.3.2.2 HPLC Analysis

Standard curves for 2-AF and 2-AAF were constructed by injecting a known concentration of each chemical as a standard versus the peak area produced by each standard. An analytical, reversed-phase, C-18 ultrasphere column was used. 20 µl of the supernatant from each reaction was injected into the column and the conversion of 2-AF to 2-AAF was monitored at 285 nm at a flow rate of 1.5 ml/min. A methanol gradient (0-100%) was used with a mobile phase (0.02 M sodium acetate, 0.2 M acetic acid, methanol, acetonitrile and H_2O (50 + 3.6 + 15 + 4 + 27.4%), pH 4.9. Under these conditions, the retention time for 2-AF and 2-AAF were 25.2 and 26.4 min, respectively (Figures 12, 13 and 14).

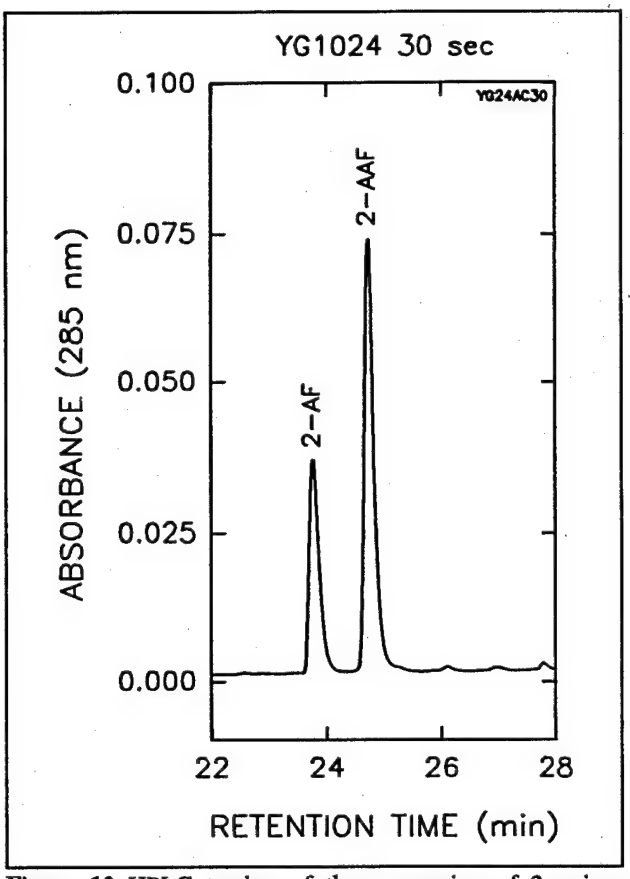


Figure 12 HPLC tracing of the conversion of 2-amino-fluorene to 2-acetylaminofluorene by YG1024 cell free extract (30 sec incubation).

2.3.3 Results

The reaction rate of acetyltransferase from each strain was calculated as the amount of 2-AAF produced per mg protein per min of incubation. The acetyltransferase reaction rates (nmole 2-AAF/mg protein/min) were 0.24±0.08, 0.71±0.06, and 320.28 ±18.9 for TA98-1,8-DNP (Figure 15), TA98, (Figure 16) and YG1024 (Figure 17), respectively. For YG1029, TA100 and TA100:OAT- the reaction rates were 244.33±57.14, 1.585±0.33 and undetectable, respectively. The reaction rates for DJ400 and DJ460 were 5817.59±273.74 and 38.11±4.84, respectively.

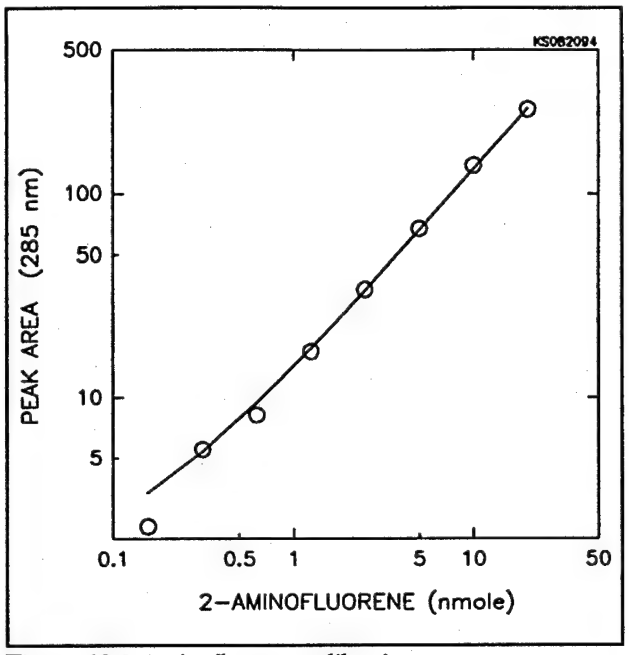


Figure 13 2-Aminofluorene calibration curve.

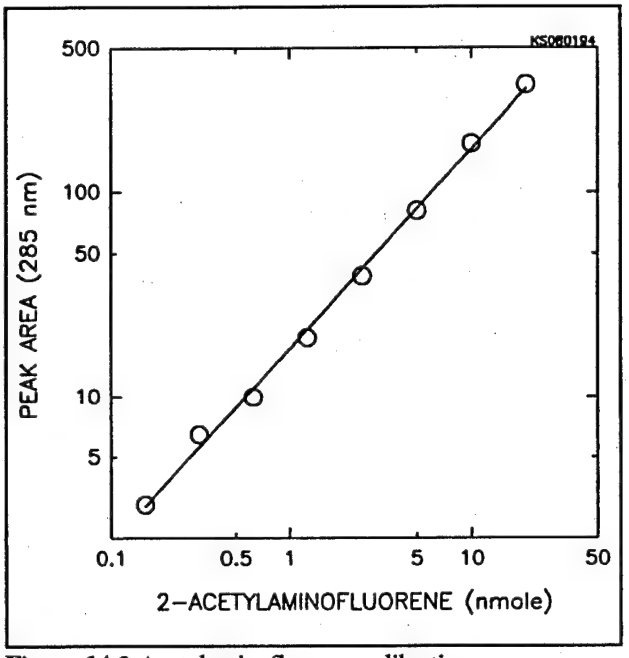


Figure 14 2-Acetylaminofluorene calibration curve.

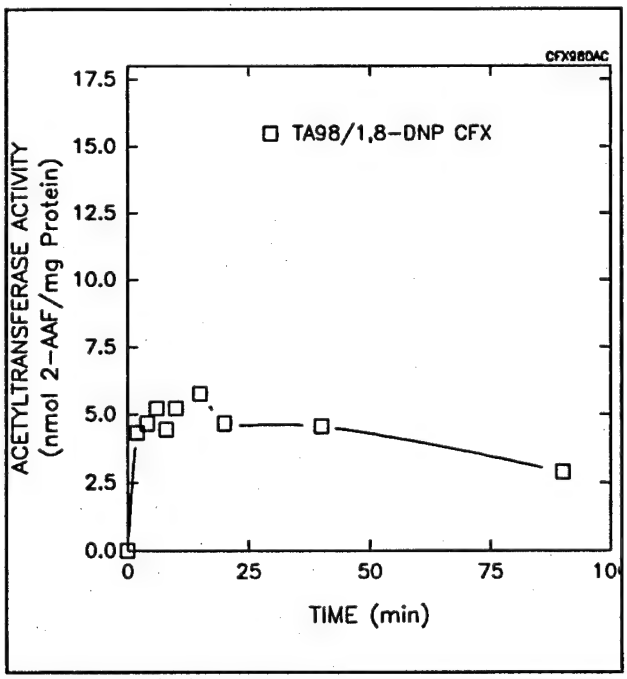


Figure 15 Kinetic analysis of the *O*-acetyltransferase activity of *S. typhimurium* strain TA98/1,8-DNP$_6$ cell free extract.

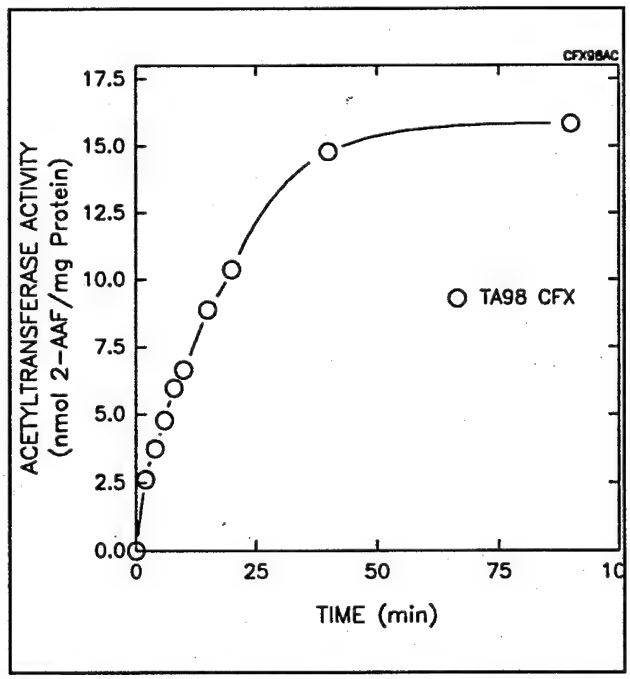


Figure 16 Kinetic analysis of the *O*-acetyltransferase activity of *S. typhimurium* strain TA98 cell free extract.

2.3.4 Discussion

The first enzymatic step in the activation of aromatic amines involves *N*-hydroxylation of the aryl ring moiety. The bacterial *O*-acetyltransferase plays a key role to further metabolize these intermediates into the ultimate mutagens. Three of the tester strains, YG1024, TA98 and TA98-1,8-DNP only differ in the copy number of the OAT gene. YG1024 has a multicopy plasmid pYG219 which encodes the OAT gene. TA98 has only one copy of the OAT gene on its chromosome while TA98-1,8-DNP is OAT-deficient. As expected, YG1024 has the highest level of OAT activity and is much more sensitive to the activation of promutagenic aromatic amines. In mammals, CoASAc-dependent arylamine *N*-acetyltransferase catalyzes the same type of enzymatic reaction as bacterial OAT and also plays an important role in the activation of *N*-hydroxyl aromatic amines. However, the substrate specificity and substrate affinity have been known to differ among these two types of acetyltransferases.

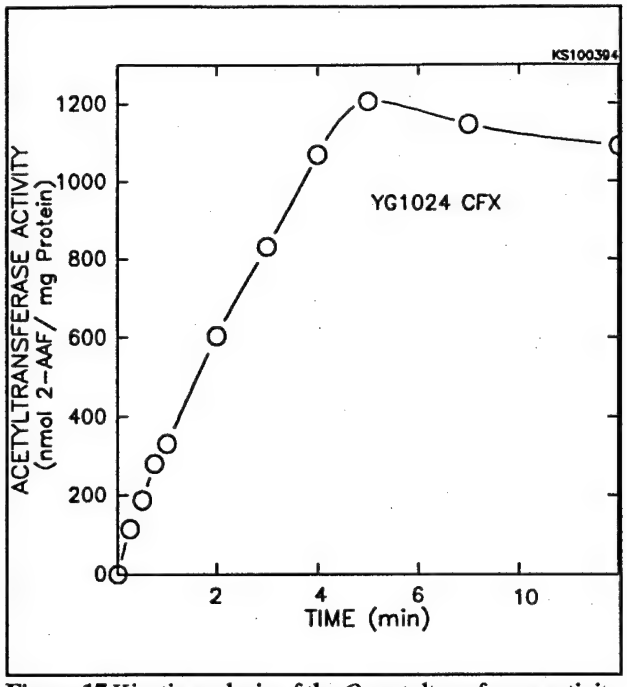


Figure 17 Kinetic analysis of the *O*-acetyltransferase activity of *S. typhimurium* strain YG1024 cell free extract.

2.4 Synergistic Genotoxic Responses Between an Organophosphorus Ester Insecticide and Arylamine Promutagens after Mammalian- or Plant-Activation

2.4.1 Introduction

We recently discovered that paraoxon and *m*PDA interact synergistically under conditions for mammalian microsomal activation or plant-activation to generate an enhanced mutagenic response in *S. typhimurium* strains TA98 and YG1024. Paraoxon (diethyl-*p*-nitrophenylphosphate, CAS Nº 311-45-5) is the toxic, but non-mutagenic metabolite of the organophosphorus ester insecticide parathion (Figure 18). Organophosphorus ester insecticides are globally employed and are environmental contaminants. *m*PDA is a monocyclic promutagenic arylamine and is a ubiquitous global contaminant. Our observation that these agents can induce a synergistic mutagenic response suggests their environmental toxicity (and perhaps the toxicity of these chemical classes) may be underestimated. This enhanced genotoxic phenomenon associated with agents that are global environmental contaminants raises concerns about their impact upon the public health. We are investigating this synergy to determine if it represents a general interaction between organophosphorus esters and arylamines.

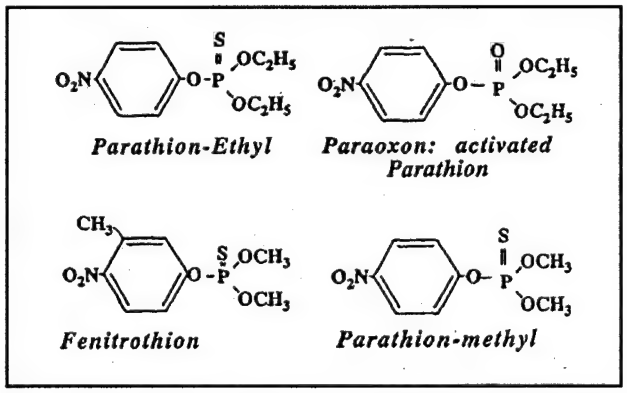


Figure 18 Chemical structures of parathion, paraoxon and related organophosphorus ester insecticides.

2.4.1.1 Paraoxon

- Paraoxon is the toxic, but non-mutagenic metabolite of the organophosphorus ester insecticide parathion (Figure 18).

- Exposure is usually by percutaneous, inhalation or oral intake.

- The agent is transported throughout the body in the blood and is activated to the toxic oxon form in the liver by cytochrome P-450 monooxygenases that convert the P=S to P=O.

- The primary toxicity of paraoxon is due to its binding to acetylcholinesterase. This enzyme mediates the degradation of the neural transmitter acetylcholine. Paraoxon interferes with the controlled degradation of acetylcholine causing unregulated continuous stimulation of the target muscles which leads to death.

- Although parathion was banned for most uses in the U.S., exposure to the insecticide is common. In 1992 over 80 restaurants in the Chicago metropolitan area were illegally treated with parathion. Parathion-methyl and fenitrothion are widely used insecticides (Figure 18).

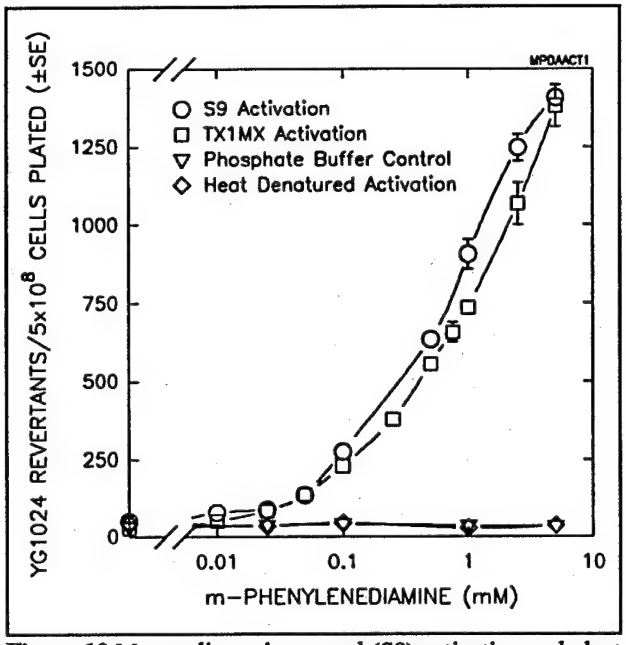


Figure 19 Mammalian microsomal (S9) activation and plant activation (TX1MX) of *m*PDA.

2.4.1.2 *m*-Phenylenediamine

- *m*PDA is a monocyclic promutagenic arylamine and was listed by the U.S. Environmental Protection Agency as a ubiquitous global contaminant.

- Arylamines are classical environmental promutagens and procarcinogens and have been used to resolve questions involving mutagenic activation, cancer induction and human polymorphic sensitivity to environmental agents.

- These agents are substrates for mammalian activation by the cytochrome P-450-mediated pathway as well as by cellular peroxidases such as prostaglandin H synthase or by plant peroxidases (Figure 19).

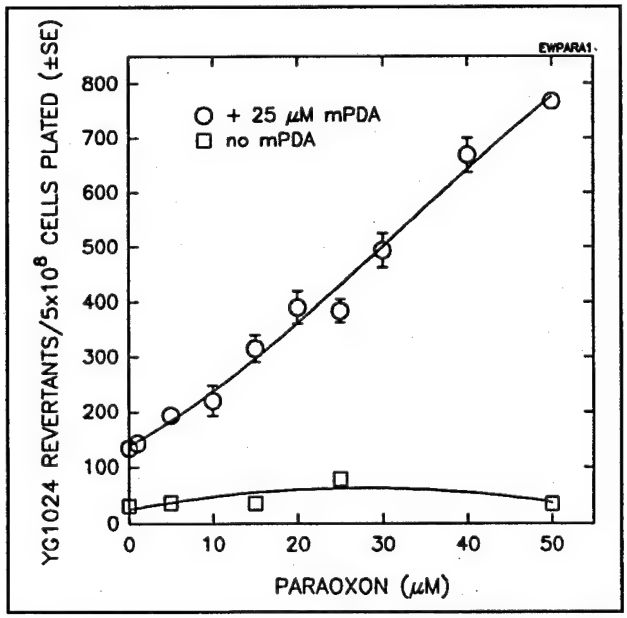


Figure 20 Mutagenic synergy with plant-activated *m*PDA and paraoxon.

2.4.1.3 *m*-Phenylenediamine + Paraoxon Mutagenic Synergy

Using a cell-free plant activation mixture (TX1MX) 25 μM *m*PDA was activated to a mutagen in strain YG1024. Each reaction tube contained 2 ml of TX1MX, 2.5 $\times$ 10^9 *S. typhimurium* cells, and 25 μM *m*PDA for a total volume of 2.5 ml. The tubes were incubated at 28° C for 1 h while shaking. From each reaction tube, triplicate 500-μl aliquots were removed and each added to 2 ml of molten top

agar supplemented with 550 μM histidine + biotin. The top agar was poured on VB minimal plates. The plates were incubated at 37° C for 72 h and the *his*$^+$ revertants scored. The mutant yield of the negative control was 37 revertants/5 $\times$ 10^8 cells plated and with 25 μM *m*PDA the frequency rose to 134.3 revertants/5 $\times$ 10^8 cells plated. When the concentration of *m*PDA was held constant at 25 μM and paraoxon was added in a concentration range of 1-50 μM a concentration-dependent increase in the mutant yield was observed (Figure 20 O). Throughout the concentration range, paraoxon was not mutagenic with or without TX1MX activation (Figure 20 □).

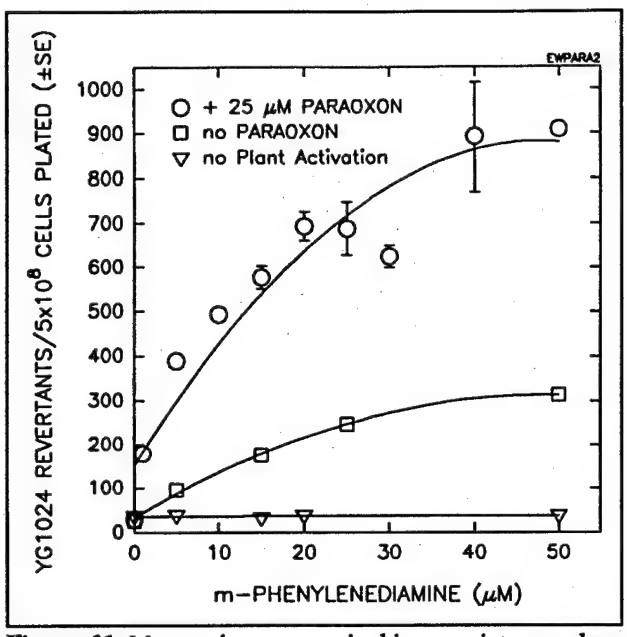


Figure 21 Mutagenic synergy in binary mixtures where paraoxon was held constant at 25 μM and *m*PDA was titrated in a range of 1-50 μM.

A reciprocal experiment to that illustrated in Figure 21 was conducted where the concentration of paraoxon was held constant at 25 μM and the concentration of *m*PDA was varied (Figure 21). The mutagenic synergistic effect is resolved by comparing the concentration-response curve of TX1MX-activated *m*PDA without paraoxon (Figure 21 □) with the curve representing *m*PDA + 25μM paraoxon (Figure 21 O). The toxicity of the paraoxon + *m*PDA mixture to the YG1024 cells was also determined. A 100-μl aliquot was isolated from each reaction tube and diluted in phosphate buffer. After an appropriate dilution series the suspension was plated onto LB medium. These plates were incubated for 24 h and the resulting colonies were counted. No alteration in the percent survivorship of YG1024 was detected (data not shown).

We also studied the effect of mammalian-microsomal S9-activation on the *m*PDA+paraoxon combination (Figure 22). Aroclor-induced rat hepatic S9 was purchased from MolTox Inc. and a 10% S9 mix was prepared. The concentration of *m*PDA was held constant at 500 μM (a 10$\times$ increase over the highest paraoxon concentration). Low concentrations of paraoxon (2.5 - 50 μM) had a dramatic synergistic effect on the mutagenicity of S9-activated *m*PDA (Figure 22A). However, there was little increased mutagenicity at paraoxon concentrations above 25 μM. This plateau in the concentration-response curve may be due to a rate-limiting component and may be the maximum synergistic response under these conditions. Throughout this concentration range of paraoxon no toxicity was observed (data not shown). A clear synergistic mutagenic response between these agents is observed using mammalian microsomal activation. A negative control of S9 and paraoxon in a concentration range of 50 - 400 μM did not induce any increase in the spontaneous frequency (Figure 22B).

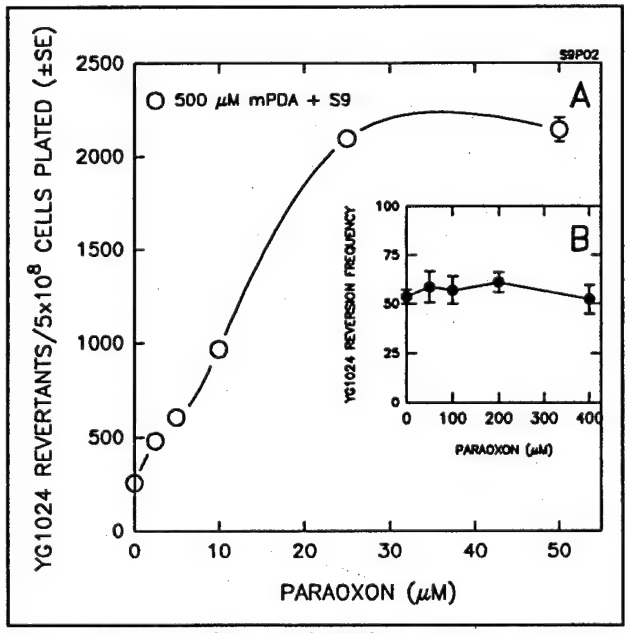


Figure 22 Mammalian S9-dependent *m*PDA + paraoxon mutagenic synergy.

2.4.1.4 Effect of Paraoxon on the Influx of a Toxic Chemical into *S. typhimurium* Cells

One hypothesis on the mechanism of the paraoxon mutagenic synergy is that the *S. typhimurium* cells may be made more permeable and allow a greater amount of mutagen into the cell. The effect of paraoxon on the toxicity of crystal violet was determined to measure if paraoxon made the cell more receptive to this agent. Figure 23 illustrates that paraoxon does not enhance the toxicity of crystal violet to strain YG1024.

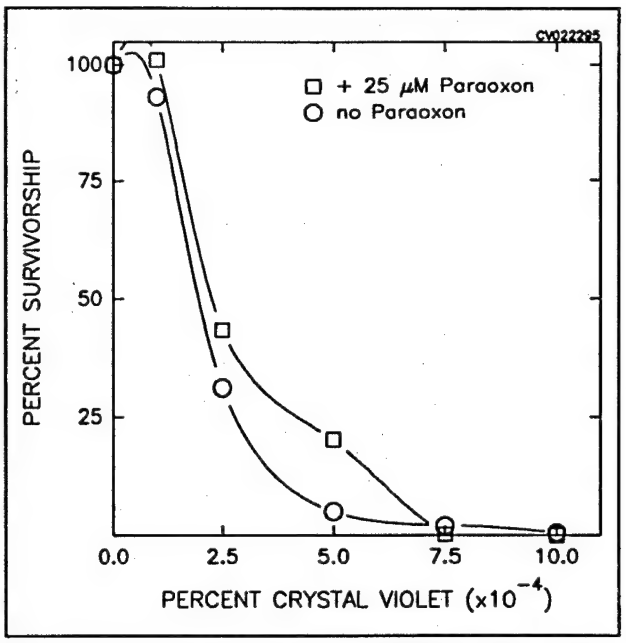


Figure 23 Survivorship curves of YG1024 with concentrations of crystal violet with and without paraoxon.

2.4.1.5 Directing-Acting Mutagens Exhibit the Paraoxon Mutagenic Synergy

A direct-acting polycyclic arylamine derivatives demonstrated a mutagenic synergistic response with paraoxon (Figure 24). 2-acetoxyacetylaminofluorene (2AAAF) is a direct-acting compound that induces a high level of reversion in YG1024. Paraoxon, at non-lethal doses (Figure 24 ○), in a concentration range from 0 to 320 μM affected an approximately 5× increase in the mutagenic response of 1 μM 2AAAF (Figure 24 △). Thus, direct-acting polycyclic arylamine metabolites also are responsive to the synergistic effect induced by paraoxon.

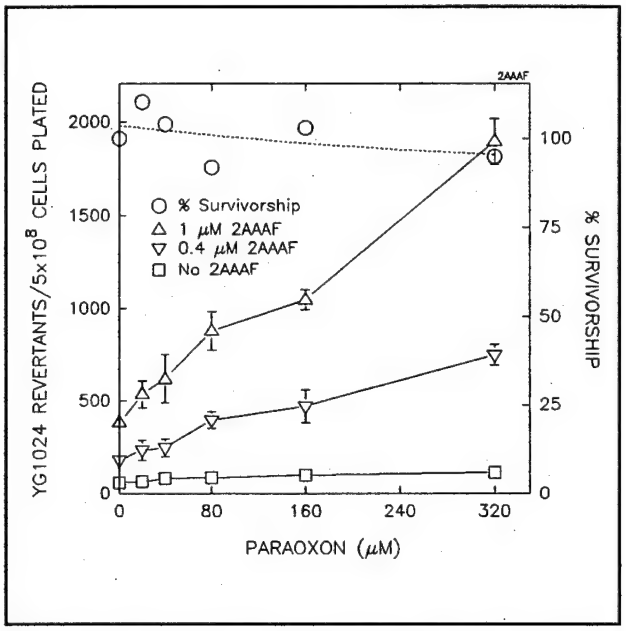


Figure 24 2AAAF + paraoxon mutagenic synergy is illustrated at 0.4 μM and 1 μM 2AAAF concentrations, (▽ and △, respectively). Paraoxon alone did not express a mutagenic response (□). The 2AAAF + paraoxon concentrations were not toxic to *S. typhimurium* cells (○).

2.4.2 Conclusions

This research demonstrates a synergistic mutagenic response between paraoxon and *m*PDA or 2AAAF.

- Either peroxidase based plant-activation (Figures 20, 21) or cytochrome P-450 based mammalian hepatic microsomal S9 activation (Figure 22) are necessary to generate the synergistic response between paraoxon and *m*PDA.
- Paraoxon is not a mutagen or a promutagen (Figure 20 □; Figure 22 ●; Figure 24 □).
- Paraoxon is acting as a co-mutagen (because it is not genotoxic) by significantly enhancing the mutagenicity of *m*PDA (Figures 20 ○) or 2AAAF (Figure 24 △,▽).
- There does not appear to be a direct mole:mole relationship between the two agents. If such a relationship existed then the mutagenic response should have reached a plateau after the paraoxon concentration exceeded 25 μM (the *m*PDA concentration was fixed at 25 μM; Figure 20 ○).
- The synergistic effect of paraoxon and plant-activated *m*PDA does not alter the mutagenic specificity.

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b. ADDRESS *(include Zip Code)*	d. AWARD DATE *(YYMMDD)*	b. ADDRESS *(include Zip Code)*	d. AWARD DATE *(YYMMDD)*	4. REPORTING PERIOD *(YYMMDD)*
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			UNITED STATES		FOREIGN		YES	NO
			YES	NO	YES	NO		
none								

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ii. NAME OF EMPLOYER	ii. NAME OF EMPLOYER		
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DATE *(YYMMDD)*

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